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THE LIPASE OF BACILLUS TUBERCULOSIS AND OTHER BACTERIA.*

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. III.

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The study of the fats and lipoids of bacteria calls attention to the presence of enzymes within the bacteria which may act upon substances of this class. That living, growing bacteria cause splitting of fats is shown beyond question by the decomposition of fats and butter by bacteria, but the particulars of the process seem to have received but little attention. The chief work on this subject was done by Eijkman,¹ who devised a simple plate method for studying the effects of various bacteria upon different substances, among them the higher fats. According to the evidence of this method, which is of necessity crude and gives only gross results, beef tallow is split into fatty acids and glycerin in the vicinity of colonies of *B. pyocyaneus*, *Staphylococcus pyogenes aureus*, *B. fluorescens* and *B. prodigiosus*; but not by colonies of *B. anthracis*, *coli communis*, *typhosus*, *diphtheriae*, *mallei*, *pestis*, *dysenteriae* (Kruse), or by cholera vibrios and several common saprophytes. More recently this work has been extended by Söhngen who used the original "auxanographic" method of Eijkman and a modification of the same.² To the foregoing list of lipolytic bacteria he adds a few more forms, all non-pathogenic. According to his observations several of the fat-splitting bacteria seem to produce two lipases. One of these, α -lipase, diffuses more rapidly and splits fat in either acid or alkaline medium; the other, β -lipase, is formed in an acid medium but does not split fats in it. H-ions inhibit and OH-ions increase the decomposition of fats in culture, and 0.02*N* acid completely inhibits lipolysis. Bacterial lipases show a great

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¹ *Centralbl. f. Bakt.*, I. Abt., 1901, 29, p. 841.

² *Kon. Akad. Wetenschappen*, 1911, 13¹, pp. 667 and 1200.

similarity in behavior to pancreas and liver lipase, and he found them capable of synthesizing fat from oleic acid and glycerol, the monoglyceride being chiefly formed. In *B. pyocyaneus*, *fluorescens*, and *liquifaciens* there is a lipase which resists heating to 100° C. for five minutes.¹ In a few of these experiments, Söhngen used extracts or killed cultures of bacteria, showing that lipases actually exist in the cultures.

Beyond the above studies by the Dutch bacteriologists there is, so far as we can find, little else in the literature bearing upon the presence of lipase in bacteria. Fuhrmann, in his *Vorlesungen über Bakterienenzyme*, gives a few references to examples of the splitting of fats by living bacteria, e.g., making butter rancid. In most of these experiments the results merely demonstrate that during bacterial growth fats may be split, but, with the exception of a few of Söhngen's experiments, do not show whether this is the result of a true lipase or some other agency, e.g., alkalies. Schreiber,² indeed, did try to demonstrate the presence of lipase by growing mass cultures of a fat-splitting bacillus in peptone water, and using this culture in 1-1000 thymol water. This killed culture exerted no splitting effect on almond oil. Fuhrmann objects to this experiment on the ground that in molds and other higher fungi there are lipases which can only be demonstrated after the cells have been shattered; but under the conditions of the experiment there should be enough disintegration of the cells by autolysis to liberate any existing lipase. We should object more to the choice of almond oil as an indicator of lipase action, since such insoluble oils offer so little surface for attack that lipolysis is very slow and of little extent, and the resulting free fatty acid is difficult of demonstration by titration in aqueous solution. As for the presence of lipase in *B. tuberculosis*, there is almost nothing known. In 1901 Carrière³ reported that broth cultures of the tubercle bacillus cause acid formation in monobutyrim. The action is destroyed by heat, but not by chloroform or ammonium fluoride, hence he concludes that it must be due to a true lipase, or a monobutyrase similar to that found by Hanriot in animal tissues. While this conclusion

¹ Söhngen, *ibid.*, 1911, 20, p. 126.

² *Arch. f. Hyg.*, 1902, 41, p. 328.

³ *Compt. rend. Soc. de Biol.*, 1901, 53, p. 320.

is probably correct, yet the evidence is unconvincing, since the titrations were performed with sodium carbonate, with phenolphthalin as indicator, so that at least the quantitative value of the results is nothing. Also, monobutyrim is not a conclusive indicator for the presence of typical fat-splitting enzymes.

Further than the above brief report we can find no statements concerning the lipolytic activity of *B. tuberculosis*. Since this organism is characterized by its power of forming and storing within itself fatty substances, and since, as previously explained, the fats of the tubercle bacillus seem of great importance in the chemotherapy of tuberculosis, we have investigated the lipolytic and ester-splitting capacity of this organism and compared it with other bacteria.

Attempts to determine the ability of tubercle bacilli to cause splitting of tallow in plate cultures, according to Eijkman's method, were unsuccessful. As this method is, at the best, not proof of the existence of lipase in bacterial cells, we did not continue these experiments exhaustively, especially when we found that organisms giving negative results by the plate method may yet possess considerable lipolytic power. Direct experiments were performed as follows:

Large quantities of the bacteria to be tested were grown on agar in flat bottles, the growth from 40 to 80 such bottles, each with a surface of about 15×5.5 cm. being used in each experiment. After a maximum growth had been reached, the bacteria were scraped off into toluene water, or washed off with toluene water, and kept at room temperature for 24 hours or longer, with an excess of toluene, and thoroughly agitated at intervals. This killed all the bacteria, as was shown by the cultivation experiments, summarized in Table 1, and also allowed a certain amount of autolysis to permit of the liberation of endocellular enzymes.

These tests show that none of the nonsporulated bacteria can survive 24 hours in toluene water, and certainly guarantee the absence of any active growth in such a medium after that period. Samples of the emulsion of tubercle bacilli used in our experiments were injected into guinea-pigs in doses containing 0.02 and 0.13 mg. of bacilli which had stood in the toluene water 20 hours and five days. None of the animals developed tuberculosis from these large doses, showing that in our experiments we were dealing with dead bacilli only.

Samples of the emulsions were taken and the dry weight of the contained bacteria determined. The emulsions were then divided into equal samples; one of each pair being heated for one-half hour submerged in boiling water to destroy all thermolabile enzymes and thus serve as a control. To each pair of samples was then added a portion of the ester to be tested, olive oil, ethyl-butyrate, or triacetin (glycerol triacetate), a slight excess of toluene, and a few drops of phenolphthalin solution; the mixture was

made neutral with $n/10$ NaOH, tightly stoppered and put in the incubator. At intervals the mixture was brought to the neutral point, and in the tables below are given the amount of $n/10$ alkali in cubic centimeters required for this purpose. In the case of actively lipolytic bacteria, part of the acidity was neutralized by $n/1$ alkali to prevent excessive dilution.

TABLE 1.
GERMICIDAL ACTION OF TOLUENE AT INCUBATOR TEMPERATURE.

TIME	<i>B. Prodigiosus</i>			<i>B. Subtilis</i>			<i>B. Coli</i>			<i>Staph. Aureus</i>			<i>B. Pyocyaneus</i>		
	Selenium* 1:50,000	Broth	Plain Agar	Selenium	Broth	Agar	Selenium	Broth	Agar	Selenium	Broth	Agar	Selenium	Broth	Agar
10 minutes at room temperature	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30 minutes incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2 hours incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6 hours incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24 hours incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
48 hours incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4 days incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10 days incubation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Selenium in a dilution of 1:50,000 was used in broth as an index of the presence of living bacteria, which cause reduction (Gosio, *Ztschr. f. Hyg.*, 1905, 51, p. 65).

Samples of each emulsion were dried and weighed, the solid substance, consisting almost entirely of bacterial cells being, in each experiment, as follows: *B. coli*, 0.945 gm.; *B. dysenteriae* (Flexner), 0.81 gm.; *Staph. pyogenes aureus*, 1.140 gm.; *B. pyocyaneus*, 0.75 gm.; with *B. tuberculosis*, there was used in each experiment two grams of fresh bacilli or about 0.22 gm. dry weight of bacilli, washed free from all other substances.

TABLE 2.
OLIVE OIL, 4 C.C.

	2 Days	5 Days	8 Days	10 Days	14 Days	Ether Extract*	Total
<i>B. coli</i> , heated	0.0	0.0	0.0	0.0	0.0	0.73	0.73
" " fresh	2.50	1.82	1.55	0.50	1.72	9.45	15.54
<i>B. dysenteriae</i> , heated	0.0	0.0	0.0	0.0	0.0	0.60	0.60
" " fresh	2.18	0.80	0.92	0.0	0.50	3.20	7.60
<i>B. pyocyaneus</i> , heated	0.0	0.0	0.0	0.0	0.0	1.42	1.42
" " fresh	24.46	14.50	4.15	3.90	2.35	18.86	68.22
" " fresh, no oil	1.70	1.90	1.40	1.10	1.51		7.61
<i>Staph. pyogenes aureus</i> , heated	0.0	0.0	0.0	0.0	0.0	0.45	0.45
" " fresh	4.65	2.0	0.0	0.0	0.0	17.70	24.35
<i>B. tuberculosis</i> , heated	0.0	0.0	0.0	0.0	0.10	0.88	0.98
" " fresh	0.0	0.0	0.0	0.0	0.0	2.68	2.68
Water and toluene	0.0	0.0	0.0	0.42	0.10	0.94	1.46

* On account of the tendency of the olive oil suspensions to form soapy emulsions which would not titrate well, and because the fatty acids liberated are held in the emulsion in such a way that the watery alkaline solution does not reach them readily, the titrations in the aqueous suspensions are not at all accurate. After the 14 days incubation the emulsions were shaken out with neutralized ether, and the fatty acids titrated with alcoholic NaOH and HCL, the resulting figure giving the amount of $n/10$ alcoholic NaOH solution required to neutralize.

TABLE 3.
ETHYL BUTYRATE, 8 C.C.

	2 Days	5 Days	8 Days	10 Days	14 Days	Total
<i>B. coli</i> , heated.....	2.80	3.02	2.75	2.80	3.08	14.45
" " fresh.....	13.00	13.12	11.35	9.88	10.62	57.97
<i>B. dysenteriae</i> , heated.....	2.97	3.58	4.10	4.42	4.80	19.87
" " fresh.....	16.20	15.91	17.60	24.43	24.30	98.44
<i>Staph. pyogenes aureus</i> , heated.....	4.85	6.15	6.15	6.30	4.00	27.45
" " fresh.....	122.80	62.85	38.82	35.00	34.50	293.97
<i>B. pyocyaneus</i> , heated.....	3.20	4.20	3.65	3.60	4.00	18.65
" " fresh.....	52.28	78.40	62.85	51.00	43.90	288.43
<i>B. tuberculosis</i> , heated.....	0.81	1.27	1.08	0.89	1.10	5.05
" " fresh.....	8.92	4.26	2.30	2.46	2.44	20.38
Water and toluene.....	0.60	0.70	0.80	0.74	0.78	3.62

TABLE 4.
TRIACETIN, 4 C.C.

	2 Days	5 Days	8 Days	10 Days	14 Days	Total
<i>B. coli</i> , heated.....	6.00	7.85	6.80	6.50	8.0	35.15
" " fresh.....	19.50	19.25	15.35	13.98	15.18	83.26
<i>B. dysenteriae</i> , heated.....	4.20	4.95	6.25	7.60	5.90	28.90
" " fresh.....	27.75	29.25	30.17	47.50	37.15	171.82
<i>Staph. pyogenes aureus</i> , heated.....	7.15	8.35	9.32	10.80	9.00	44.62
" " fresh.....	142.35	182.00	70.00	60.00	25.00	479.35
<i>B. pyocyaneus</i> , heated.....	7.50	10.20	7.90	8.30	9.50	43.40
" " fresh.....	73.00	77.20	53.00	26.50	25.00	254.70
<i>B. tuberculosis</i> , heated.....	1.83	1.97	2.31	3.16	4.62	13.89
" " fresh.....	10.55	9.88	9.03	11.49	20.35	61.30
Water and toluene.....	1.45	2.07	2.11	2.40	4.88	12.91

Inspection of these tables shows at once a definite hydrolytic activity on the part of all the bacteria studied. While *B. pyocyaneus* and *Staphylococcus pyogenes aureus*, which are classed by Eijkman as among the fat-splitting bacteria according to the plate method, show by far the most active lipolysis, yet *B. dysenteriae* and *B. coli*, which do not split tallow under agar plate colonies, are possessed of no inconsiderable lipolytic activity. From this it seems safe to conclude that the plate method as heretofore used shows only the most active degrees of lipolysis, and is solely a relative test for lipase. Bacteria not giving positive results by this method may be, nevertheless, possessed of considerable lipolytic activity. It is, indeed, highly improbable that any living cells are

devoid of lipolytic activity, whether animal, plant, or bacterial cells. A possible source of error might be imagined if bacteria undergoing autolysis produced acids from other sources than fats, e.g., lactic acid from carbohydrates. This possibility is eliminated by the experiments with olive oil, which show that almost no water-soluble acid is produced by these bacterial suspensions during autolysis except by *B. pyocyaneus*, and here the amount is relatively very insignificant.

It is noticeable that a certain amount of hydrolysis is shown by the cultures which have been heated for 30 minutes at approximately 100° C.; also, that this varies in a measure in direct proportion to the hydrolytic activity of the unheated killed bacteria, as if it represented a residual portion of the thermolabile enzyme. Without attempting to analyze further or to interpret this observation, it may be compared with the statement of Söhngen that certain bacteria possess a lipase resisting 100° C. for five minutes, which he interprets as a special thermostabile lipase.

As for *B. tuberculosis* it is apparent that, in common with all its biological properties, it is not an active splitter of fats and simpler esters; but these experiments do demonstrate that it does possess enzymes capable of slowly hydrolyzing the three very different esters used in these experiments. However, it probably falls short in activity of *B. coli* and *B. dysenteriae*, which are among the least actively lipolytic of the rapidly growing pathogenic bacteria, but presumably the low figures are at least partly ascribable to the use of smaller quantities of bacillary substance. There are too many complicating factors in the way of bacterial fats, adventitious substances, etc., in ascertaining the quantity of bacterial substance used to make our results of much quantitative value.

One noticeable fact is that each bacterium exerts a corresponding effect on each of the three sets of esters used, the staphylococcus being most active in hydrolyzing each, pyocyaneus coming second, colon, dysentery, and tubercle bacilli following in order. This suggests that the same enzyme is concerned in each case, which seems more probable than the assumption that each ester is hydrolyzed by a specific enzyme and that all three enzymes are developed to a corresponding degree in each of the five species.

Kastle, Loevenhart, Amberg, and Peirce¹ have directed attention to the extreme susceptibility of the lipases of animal tissues to fluorides, so that marked inhibition of ester splitting is caused by dilutions of NaF as high as 1-500,000 or more. This inhibition is most marked with esters of the lower fatty acids, decreasing as one ascends in the fatty acid series of esters. Experiments were, therefore, performed to discover whether bacterial lipases are similar to animal tissue lipases in this respect.

In the first series, the growth on 80 bottles of a 48-hour culture of *B. pyocyaneus* was suspended in 420 c.c. toluene water, and the dried weight of bacterial substance present in 10 c.c. samples was found to be 0.203 gm. Samples of 50 c.c., containing each 1.015 gm. of bacterial substance, were then used for the experiments, to each being added sufficient 1-1000 NaF solution to make the strengths indicated, the total amount of fluid being made approximately the same in each sample. Because of the large amount of acid developed, the titration was first made approximately with normal alkali, to avoid too great dilution and then finished with $n/10$ solutions, the figures given being for $n/10$ NaOH. After each titration, sufficient NaF solution was added to hold the fluoride concentration at approximately the given figure. In order to ascertain the influence of the amount of enzyme on these results, in the second experiment but 10 bottles of culture were used, giving 0.145 gm. of bacterial substance for each sample. Here the titrations were made with $n/4$ solutions, but the figures are given for $n/10$ alkali as in all the other experiments.

These experiments indicate that the lipase of *B. pyocyaneus* behaves under the influence of sodium fluoride exactly as Loevenhart and his co-workers found the lipase of liver and pancreas to behave. In Series A during the first three days there is a decided inhibition of the hydrolysis, but after this time the degree of inhibition becomes less and less marked, until at the end of two weeks there may be even more rapid hydrolysis in the presence of the fluoride. Series B, in which was used a much smaller amount of bacterial substance, shows a similar tendency, which appears later.

¹ Loevenhart and Peirce, *Jour. Biol. Chem.*, 1907, 2, p. 397; and Amberg and Loevenhart, *ibid.*, 1908, 4, p. 149.

This behavior agrees perfectly with the findings of the above authors, and is ascribable to a number of factors. One is the accumulation in the solution of the sodium salt of the acid formed by hydrolysis, which Amberg and Loevenhart found to counteract the effects of the fluoride. Another is their observation that the fluoride causes final equilibrium to be reached more slowly, so that after the first rapid hydrolysis without the presence of fluoride

TABLE 5.
EXPERIMENT A. 1.015 GM. *B. Pyocyaneus*.

	1 Day	3 Days	6 Days	10 Days	14 Days	17 Days
<i>Triacetin</i> —						
NaF, 1-3,000.....	46.9	68.7	73.0	60.0	37.2	30.8
NaF, 1-37,500.....	69.1	97.2	85.0	62.0	37.5	29.6
NaF, 1-150,000.....	83.5	117.4	79.0	62.0	39.5	29.8
Control.....	90.4	119.5	74.0	59.5	35.5	28.4
<i>Ethyl butyrate</i> —						
NaF, 1-3,000.....	12.8	18.5	15.4	10.7	14.6	10.7
NaF, 1-37,500.....	16.4	14.0	15.0	11.5	13.8	10.9
NaF, 1-150,000.....	16.5	23.5	18.7	8.3	8.0	8.8
Control.....	17.0	20.0	16.0	8.3	9.8	8.2

EXPERIMENT B. 0.145 GM. *B. Pyocyaneus*.

	1 Day	3 Days	6 Days	10 Days	14 Days	17 Days
<i>Triacetin</i> —						
NaF, 1-3,000.....	11.0	16.2	19.6	33.4	33.6	40.3
NaF, 1-37,500.....	13.4	24.1	34.5	46.4	44.0	56.1
NaF, 1-150,000.....	19.0	30.0	47.3	53.7	41.5	57.0
Control.....	22.3	38.8	48.8	56.0	43.8	51.2

the digest which has been held back by the fluoride may then show more active hydrolysis during a given period of time. Also, the accumulation of acid in the digest injures the lipase, and the reduction in acid formation by fluorides will also decrease the injury to the lipase from this source and tend to keep it active for a longer period. The observations of Loevenhart and Peirce, that hydrolysis of triacetin by animal lipase is more depressed by fluorides than the hydrolysis of ethyl butyrate, and also that the larger the amount of enzyme the less the effect of the fluoride, are also duplicated by *pyocyaneus* lipase. It would seem that the similarity of the results is so great as to warrant the conclusion that the lipase of *B. pyocyaneus* is of the same character as the lipase of mammalian liver and pancreas.

SUMMARY.

By directly testing the killed bacterial substance upon various esters and fats, the presence in bacteria of typical lipolytic enzymes can be demonstrated, even with organisms which cause no visible splitting of fats in plate cultures (the "auxanographic" method). Bacteria causing visible fat splitting in plate cultures (*B. pyocyaneus* and *Staphylococcus pyogenes aureus*) are much more actively lipolytic than bacteria which do not give positive results by this method (*B. coli communis*, *B. dysenteriae* [Flexner], *B. tuberculosis*). All these five organisms cause hydrolysis of olive oil, ethyl butyrate, and glycerol-triacetate. The order of hydrolytic activity is the same for each of the three esters, being as follows: *Staphylococcus*, *B. pyocyaneus*, *B. coli*, *B. dysenteriae*, and *B. tuberculosis*. Presumably the same enzyme attacks all three esters. *B. tuberculosis* is probably less actively lipolytic than the other bacteria tested.

The bacterial lipases resemble the lipases of mammalian tissues in being inhibited by sodium fluoride in high dilutions. The similarity of behavior is so great as to indicate the similarity or identity of the lipases of bacteria and mammalian tissues.